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Please file the following enclosed patent application papers:

Applicant #1, Name: Robert W Brocia
Other Applicant(s):
Title: A METHOD TO DETERMINE THE ACTIVITY OF AN ENZYME

(x) Specification, Claims, and Abstract: Nr. of Sheets 17

(x) Declaration: Date Signed: December 27, 1997

(x) Drawing(s): Number of Sheets Enclosed: (In Triplicate):

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(x) Small Entity Declaration of Inventor(s)

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Very respectfully,

Signature

12/27/97
Date

Robert W Brocia
15 Moore Road Bronxville, NY 10708

Patent Application of Robert W Brocia
for
A METHOD TO DETERMINE THE ACTIVITY OF AN ENZYME

Background -- Field of Invention

This invention relates generally to the field of biochemistry and clinical chemistry. More particularly, the invention is a novel method to measure the activity of an enzyme.

Background -- Description of Prior Art

Conventional characterization of a protein with enzymatic activity is expressed in terms of product formed per mass of protein present per unit time. This is referred to as the specific activity of the protein. For example, a protein that hydrolyzes a substrate to form products X and Y may be described as having a specific activity of moles of X or Y formed per gram of protein per minute.

This specific activity determination requires two separate laboratory procedures 1) a measurement of the amount of product formed (moles) in a certain amount of time by a sample of the protein, and 2) an assay to determine the protein mass (gms) present in the sample. Normally the protein mass is measured in a colorimetric assay such as a lowry protein assay or a BCA assay. The mass determination requires that a portion of the protein be sacrificed so that it may be reacted with the lowry reagents.

The conventional specific activity characterization is limited to proteins that have bond breaking and/or making activity such as lipases, esterases, oxidases and others. The bond breaking and/or making activity is defined herein for ease of explanation of the invention as activity that makes and/or breaks chemical bonds.

The present invention is useful for characterization of these proteins and other proteins that do not make and/or break bonds. For example, proteins such as cholesteryl ester transfer protein (CETP), microsomal [triglyceride] transfer protein (MTP), phospholipid transfer protein (PLTP) do not cleave a chemical bond. These proteins express their activity by transporting lipids among donor/acceptor sites, it is believed that the activity of these proteins is modulated according to the liquid crystalline states of their respective substrates and may follow an entropy gradient and in addition there are proteins that do make/break bonds but are unusual in that there activity changes according to the physical state of the substrate, i.e. lipase. All these lipid active proteins CETP, MTP, PLTP and lipase and others including lecithin cholesterol acyl transferase (LCAT), ACAT and others including enzymes that react with protein substrates of varying liquid crystalline states may be characterized by the present invention. The present invention is also useful to characterize enzymes that are described by convention means.

The present invention is utilized to characterize all enzymes and normalizes the activity of the enzyme by the amount of substrate or protein mass present. The invention accomplishes this for use such as in the clinic where samples of physiological fluids may contain protein activity. The samples may demonstrate varying protein activity from patient to patient. The differences in activity solely

due to the amount of protein present in the sample or active mass must be discerned from activity due to varying amount of substrate present in the sample.

For example, cholesteryl ester transfer protein (CETP) is a plasma protein that shuttles lipids among lipoproteins (including high density lipoprotein [HDL], low density lipoprotein [LDL], intermediate density lipoprotein [IDL], very low density lipoprotein [VLDL] and chylomicrons). If the plasma CETP activity is measured in a group of patients, the measurement must take into account the concentration of lipoprotein particles present in each sample. This is conventionally achieved by adding an excess of VLDL or LDL to the test sample to normalize the acceptor concentration among each sample. The conventional CETP assay would include a volume of a patients plasma combined with a CETP compatible cholesteryl ester (CE) donor particle. The CE is labeled so that the CE mass may be quanitated after the protein shuttles the CE from donor to acceptor. A suitable acceptor is added to the plasma and donor mixture in a buffer to replicate physiological conditions. The donor, acceptor, plasma and buffer mixture is incubated. After incubation, the assay is analyzed to determine the amount of labeled CE transferred from donor to acceptor.

The added acceptor is in excess and compensates for the differences in lipoprotein particle numbers associated with each patients plasma lipoprotein profile. If the acceptor were not added the endogenous lipoproteins would accept the transferred CE and results of the test would vary based not only upon activity of the protein but also according to each patients lipoprotein profile.

The present invention improves upon the conventional methods of determining CETP activity in a plasma sample. The present invention eliminates the

acceptor of the previous method. Thus reducing variability associated with VLDL preparations and further eliminates hazards involved with the handling of human blood products. The components of the method are more stable and have a longer shelf life without VLDL.

The present invention includes a CE donor with a fluorescent label on the CE. The fluorescent cholesteryl ester is 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-23,24-bisnor-5-cholen-3-yl linoleate (NBD-CE). The CE with fluorescent label (NBD) included in the donor is formatted so as CETP activity causes a change in fluorescence over time. Furthermore, the invention includes the fluorescent label to block reaction with non-CETP proteins such as cholesteryl esterase.

Objects and Advantages

Accordingly, several objects and advantages of my invention are An enzyme assay that internally controls the assay result for factors that affect enzyme activity.

It is also a principle object of the invention to characterize a protein activity in one assay.

It is another object of the invention to characterize a protein activity in a clinical sample for the purpose of diagnosis of a disease.

Still further objects and advantages will become apparent from a consideration of the ensuing description and accompanying drawings.

Brief Description of the Drawings

Fig. 1 is an illustration that depicts the light emitting enzyme activity determination step of the assay followed by the normalization step of the assay in three different samples.

Fig. 2 shows the invention as immunoturbidometric normalization is applied.

Fig. 3 shows the invention applied to lipid transfer protein, specifically CETP.

Summary

The present invention is a method to measure protein activity that internally controls for factors that affects protein activity such as substrate concentration.

Preferred Embodiment -- Description

The present invention is a method to characterize protein activity wherein the characterization is not calculated from separately determined values such as activity and protein mass. The present invention involves an enzyme activity parameter and a normalization parameter. The activity parameter method according to the invention is a protein activity assay wherein the result, i.e. net chemiluminescence or fluorescence of the activity assay will change when an assay to determine the concentration of the protein, substrates or other factor of interest is performed if the change in the result is not due to a change in protein activity.

The present invention is a clinical diagnostic test wherein the result of the test on a sample solution is obtained in one determination from one instrument yet the result that is detected by the instrument is due to a combination of more than one independent spectrophotometric assay. One spectrophotometric assay emits light and another alters the optical density of the assay. The combined effect is measured as one result by one instrument.

A clinical diagnostic test wherein the result is measured by one instrument in one determination and the result is a function of more than one chemical indicator where each chemical indicator functions independently.

The invention is a screen for inhibitors or promoters of protein activity for the purpose of pharmaceutical drug development.

This invention disclosure provides several examples of different fluorescent activity assays where the emitted light is varied according to protein activity and then adjusted with respect to the substrate concentration or, alternatively, adjusted with respect to protein concentration by immunological techniques or colorimetric assay.

The activity parameter is established with a light emitting measurement technique that includes fluorescence and chemiluminescence enzyme activity assays where the protein activity is assessed by a fluorimeter or luminometer as a change in light emission intensity.

Preferred Embodiment -- Operation

Fig. 1 illustrates the enzyme activity dependent fluorescence or chemiluminescence portion of the method according to the invention followed by the substrate or product normalization portion of the invention. In Fig 1 enzyme and substrate interactions 10, 11, 12 are depicted so substrate concentration is increasing from 10 to 12. After enzyme substrate interaction fluorescent or chemiluminescent products 20, 21, 22 are obtained. The products are of varying fluorescent or luminescent intensity according to varying substrate concentration. Normalization factor 30,31,32 is interacted with the light emitting mixture and color develops 40, 41, 42 according to the increasing substrate concentration present. The developed color causes an increasing optical density thus modulating the light emission intensity from the mixture. The fluorimeter or luminometer or other light detecting instrument would collect the substrate dependent light emission intensity.

FIG. 2 illustrates another embodiment of the invention. Enzyme and substrate mixture 100 yields an activity dependent light emitting product 110. Normalization factor 120 is an antibody specific for the enzyme and immunoprecipitation components. Resulting mixture 130 is turbid by precipitated antibody/enzyme complex and the turbidity blocks the emitted light from the light emitting product formed by the enzyme activity. The results are light emission intensity that is normalized by enzyme mass present.

The following are specific examples involving certain proteins of interest to provide a complete understanding of the invention:

In the diagnosis of heart disease the lipid transfer proteins seem to play an important role. The many complex interactions involving lipids make a clear path for intervention difficult to perceive. For example, the relationship between saturated fats in a patients diet and the patients total plasma cholesterol is believed to be a factor of solubility. If an amount of saturated fat is packaged in a lipoprotein particle core, the lipoprotein particle core will also solubillize cholesteryl ester. Cholesteryl ester has an increasingly limited solubility in triglycerides (TG) of increasing saturation which means a fixed mass of cholesteryl ester will require more lipoprotein particles for solubilization if the triglyceride is of a more saturated type. Fewer lipoprotein particles are required if the TG is of an unsaturated type.

Therefore, a saturated fat diet will generate more lipoprotein particles to move about an equivalent amount of cholesteryl ester. Additionally, free cholesterol will partition into the lipoprotein fraction at about 3% solubility factor from the plasma red cells boosting plasma cholesterol in a saturated fat diet dependent manner.

Cholesteryl ester transfer protein (CETP) is a protein present in normal human plasma. CETP transfers lipids among lipoprotein particles. Of the most important of these transfer events is the transfer of cholesteryl esters (CE) from high density lipoprotein (HDL) to low density lipoprotein (LDL) or very low density lipoprotein (VLDL).

This example is important to express the invention in terms of an unusual enzyme such as CETP because with CETP a product is not formed by breaking chemical bonds as with other enzymes. Proteins such as cholesteryl ester transfer protein (CETP), microsomal [triglyceride] transfer protein (MTP), phospholipid transfer protein (PLTP) do not cleave a chemical bond. These proteins express their activity by transporting lipids among donor/acceptor sites, it is believed that the activity is according to an entropy gradient and in addition there are proteins that do make/break bonds but are unusual in that there activity also changes according to the physical state of the substrate, i.e. lipase. These lipid active proteins CETP, MTP, PLTP and lipase and others including lecithin cholesterol acyl transferase (LCAT), acyl cholesterol acyl tranferase (ACAT) and other enzymes not lipid active, including enzymes that react with protein substrates acording to the liquid crystalline states of the protein substrates.

Conventionally, CETP activity cannot be expressed as a specific activity because there is no product formed by making/breaking chemical bonds. The following example presents one embodiment of the present invention as a clinical method to measure CETP activity:

A suitable volume of the patents plasma is incubated with the CE donor in buffer according to the invention. The CE donor is comprised of a fluorescently labeled CE. The fluorescent label includes NBD. The fluorescence increases, in the case of NBD, over time as the plasma CETP transfers the fluorescent CE from the donor to endogenous lipoprotein particles.

The fluorescence intensities among the group of samples will be varied according to the CETP activity in the samples *and* any variability among the

concentrations of lipoproteins in samples. For example, differences among a patients LDL cholesterol will be reflected in the activity of CETP. This is explained by the LDL cholesterol values resulting from the actual number of LDL particles present in the plasma. So a patients plasma sample that is high in LDL cholesterol has more LDL particles in suspension then a patient who has a low LDL cholesterol. The patients plasma with high LDL cholesterol will appear to have a high CETP activity when in fact the apparently high activity is due to the greater number of LDL particles available to accept transfer of the NBD-CE or other fluorescent cholesterol.

Furthermore, given two patients with identical CETP activity and lipoprotein profiles, one has a meal and the other is fasted, the CETP is measured with donor and no exogenous acceptor. The fed patient will have an apparent increase in CETP activity because of chylomicron particles that circulate in the plasma after meals as a normal component of digestion. The chylomicrons will behave as acceptor of CE. The CETP activity will appear to be higher in the fed patient when in fact CETP mass may not change.

The present invention accounts for variable lipoprotein profiles by normalizing with a color development reaction in response to cholesterol and / or triglyceride and / or phospholipid and / or protein. The development of color creates a quenching effect upon the fluorescence of the CE. Therefore, the greater the concentration of CE/TG/PL/protein the greater is the color quenching effect upon the fluorescent label. This normalizes the fluorescent intensity for LDL concentration.

The present invention provides an assay that yields one value representing the activity of an enzyme. The invention accounts for enzyme specific variables that may normally affect the activity of a protein in an activity assay.

The invention is applied to the measurement of activity of CETP present in a patients plasma through the use of a synthetic donor particle. The donor particle provides a source of fluorescently labeled CE to the protein. The CE is present in a self-quenched state in the core of the donor particle. Therefore, when the CE is removed from the core by the protein a measurable increase in fluorescent occurs. The CETP shuttles the cholesteryl ester from the donor particle to endogenous lipoproteins present in the plasma sample. These endogenous lipoproteins act as acceptor particles. The more active the CETP the higher is the fluorescence after a period of time.

The patients plasma will have a variable amount of endogenous lipoprotein acceptor particles dependent upon the patients particular plasma lipoprotein profile. Therefore, the fluorescence increased from the activity of the CETP present in a patients plasma is dependent on the concentration of lipoproteins present in their plasma as well as the activity of the CETP. The invention provides a normalization factor based upon substrates of the CETP which include endogenous cholesteryl esters and triacyl glycerols, the major core components of endogenous HDL, LDL and VLDL. The normalization factor may also include phospholipids or cholesterol or any component present in the sample that would have an effect on CETP activity.

The normalization factor includes a colorizing factor that reacts in response to the normalizing factor of choice. For example, in the case of CETP, neutral

lipids (CE and/or TG) may be used as the normalizing factor. A mixture of cholesteryl esterase (CEH) >100 U/L, cholesterol oxidase (CO) 300 U/L, peroxidase (PO) 1000 U/L, 4-aminoantipyrine 0.3 mmol/L, p-hydroxybenzenesulfonate 30 mmol/L in a buffer at pH 6.5 (in the case of CE as the normalization factor) is added to the incubation comprising fluorescent CE / donor and plasma. The CEH hydrolyzes any non-fluorescent cholesteryl esters to cholesterol. The cholesterol is oxydized by CO to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide is coupled with 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of PO to yield a quinoneimine dye. The assay changes color in response to the concentration of C/CE in the plasma sample [not responding to the fluorescent CE in the donor due to the fluorescent label blocking CEH or binding but not CETP]. The higher the concentration of the C/CE from the endogenous plasma lipoproteins, the darker the color from the colorimetric assay. Increased color decreases the measurable fluorescence intensity of the activity assay due to color quenching of the fluorescent label thereby normalizing the results according to the endogenous lipoproteins present in the plasma.

Normalization for TG in the assay includes the addition of a mixture of reagents that respond to TG with a color development. For example, a mixture of reagents that include: adenosine triphosphate (ATP) 0.3 mmol/L, magnesium salt 3 mmol/L, 4-aminoantipyrine 0.15 mmol/L, sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine 1.69 mmol/L, lipase 50,000 U/L (LP), glycerol kinase 1000 U/L (GK), glycerol phosphate oxidase 2000 U/L (GPO), peroxidase (PO) and buffer at pH 7.0 is added to the assay. Triglycerides are hydrolyzed by LP to glycerol and free fatty acids. Glycerol is phosphorylated by ATP forming glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate in a reaction catalyzed by GK.

G-1-P is then oxidized by GPO to dihydroxyacetone phosphate and hydrogen peroxide. A quinoneimine dye is produced by the PO catalyzed coupling of 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine with hydrogen peroxide. The color develops according to the concentration of triglyceride present in the assay. The color affects the fluorescence intensity reading determined by the instrument.

The invention then internally normalizes CETP activity based upon CE or TG concentration of a patients plasma.

It is important to note that although the normalization components of the invention are indicator enzymes which hydrolyze specific substrates present in plasma to effect a optical density change, the indicator enzymes do not react with the fluorescently labeled cholesteryl ester of the donor particle.

The present invention is applied to lipid transfer proteins, such as CETP according to the figures as follows: in Fig. 3, a sample 300, is a plasma or other physiological sample that may include variable concentrations of lipoproteins and CETP. A volume of the sample is incubated in an appropriate buffered incubation mixture 310 with fluorescent cholesterol ester donor particle 320 comprised of self-quenched fluorescently labeled cholesteryl ester 330. During incubation CETP 340 present in the sample transfers fluorescently labeled cholesteryl ester to any acceptors 350, such as lipoproteins, present in the sample. As transfer occurs there is an increase in fluorescence intensity in the incubated sample. The increase is dependent upon the activity of lipid transfer protein and the concentration of endogenous acceptor present in the sample. The normalization factor is applied according to the invention and may include

normalization with respect to the lipid transfer protein by utilizing an immunoprecipitation technique with a CETP antibody. The normalization may be based on colorimetric techniques utilizing TG and or CE due to the presence of endogenous lipoproteins in the sample.

Conclusions, Ramifications, and Scope

Accordingly, it can be seen that the invention provides a convenient technique to characterize a protein activity in one instrument with the inclusion of at least two spectrophotometric assays.

Although the description above contains many specificities, these should not be construed as limiting the scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within it's scope. For example, virtually any protein activity may be assayed with one internally controlled assay according to the invention.

Thus the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given.

Claims: What is claimed is:

1. A method to measure enzyme activity comprising;

a protein activity dependent light emitting component, and ;

a substrate concentration dependant light quenching component.

2. The method of claim 1 wherein said protein activity dependent light emitting component includes a donor particle comprised of fluorescent lipid and said substrate concentration dependant light quenching component includes a colorimetric assay specific for neutral lipids.

3. The method of claim 1 wherein said protein activity dependent light emitting component includes a donor particle comprised of fluorescent lipid and said substrate concentration dependant light quenching component includes a turbidometric assay specific for a protein.

4. A cholesteryl ester comprised of a fluorescent label wherein said label blocks cholesteryl esterase activity and does not block cholesteryl ester transfer protein activity.

5. A method to determine enzyme activity comprising an activity parameter and a normalizing parameter.

6. The method of claim 5 wherein said activity parameter is a light emitting assay and said normalizing parameter quenches light emission.

7. The method of claim 6 wherein said normalization parameter includes turbidity from immunoprecipitation products.

8. A method to determine one clinical diagnostic value from a sample wherein said method comprises more than one spectrophotometric assay.

9. The method of claim 8 wherein said spectrophotometric assay includes an assay that increases the optical density of the sample solution.

10. A clinical diagnostic test wherein the result is measured by one instrument in one determination and said result is a function of more than one independantly functioning chemical indicator.

11. A method to assay plasma CETP activity without exogenous acceptor.

A METHOD TO DETERMINE THE ACTIVITY OF AN ENZYME

Abstract:

A method to determine the activity of a protein wherein the method internally controls for substrate concentration. The method includes a fluorescent or chemiluminescent activity assay followed by a normalization step that quenches the light detected by an instrument measuring the assay. The quenching is in response to a concentration of substrate or protein present in the assay.

Fig. 1

Fig. 7

FIG 3

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First/Sole Applicant: Robert W Brocia
Other Applicant(s):
Title: "A METHOD TO DETERMINE THE ACTIVITY OF AN ENZYME"

Small Entity Declaration - Independent Inventor(s)

As a below-named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35 United States Code, to the Patent and Trademark Office with regard to my above-identified invention described in the specification filed herewith. I have not assigned, granted, conveyed, or licensedand am under no obligation under any contract or law to assign, grant, convey, or license any rights in the invention to either (a) any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or (b) any concern which would not qualify as either (i) a small business concern under 37 CFR 1.9(d) or (ii) a nonprofit organization under 37 CFR 1.9(e).

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I acknowledge a duty to file, in the above application for patent, notification of any change in status r (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Signature

12/27/97
Date

Robert W Brocia
15 Moore Road Bronxville, NY 10708

A/Des
A

Declaration for Utility or Design Patent Application

As a below-named inventor, I hereby declare that my residence, post office address, and citizenship are as stated below next to my name and that I believe that I am the original, first, and sole inventor [if only one name is listed below] or an original, first, and joint inventor [if plural names are listed below] of the subject matter which is claimed and for which a patent is sought on the invention, the specification of which is attached hereto and which has the following title:

"A METHOD TO DETERMINE THE ACTIVITY OF AN ENZYME"

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration. I acknowledge a duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, Section 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Please send correspondence and make telephone calls to the First Inventor below.

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Date

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